Specific patterns of neuronal loss in the pulvinar nucleus in dementia with Lewy bodies

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ABSTRACT

Complex visual hallucinations occur in 70-80% of dementia with Lewy bodies (DLB) subjects and significantly affect wellbeing. Whilst the pathobiology of visual hallucinations in DLB remains poorly understood, several hypothetical models have suggested that visual attentional mechanisms may be altered, leading to a potential vulnerability to visual hallucinations. The present study investigated whether neuropathological changes occur in the pulvinar nucleus, a thalamic structure with a fundamental role in visual attention. *Post-mortem* pulvinar tissue was acquired from eight DLB, eight Alzheimer's disease (AD) and eight control cases and analyzed using stereological and quantitative neuropathological techniques. Lewy body pathology was found in all pulvinar sub-regions in DLB cases. However, neuronal loss was specifically found in the lateral pulvinar of DLB cases compared to control cases. Although significant reductions in lateral neuron number were also found in AD cases compared to controls, these changes were not as marked as those observed in DLB cases. Previous studies have shown alterations to lateral areas of the pulvinar on neuroimaging, where they were found to be related to the frequency and severity of visual hallucinations. The lateral pulvinar is thought to modulate visual cortical activity based on attentional demands, thus contributing to visual attentional functioning. As alterations to visual attentional function and visual cortical activity have been postulated to contribute to visual hallucinations, the present results suggest neuropathological changes in visual components of the pulvinar that may contribute to attentional deficits and promote the manifestation of visual hallucinations in DLB.

Keywords: Lewy, hallucination, stereology, pulvinar, thalamus, dementia

INTRODUCTION

Dementia with Lewy bodies (DLB) is the second most common form of primary neurodegenerative dementia after Alzheimer's disease (AD) (1), accounting for approximately 4.2% of all dementia cases (2). Clinically, DLB is characterized by three core symptoms of fluctuating cognition, parkinsonism and visual hallucinations, in the presence of global cognitive decline (3).

Visual hallucinations occur in 60-80% of DLB cases (4) and have been found to reduce patient quality of life (5, 6) and add to caregiver burden (7). Visual hallucinations in DLB are usually complex and recurrent, often involving animals, insects and/or disembodied faces (8). Visuo-perceptual deficits, including impairments in eye movements and complex visual functions, are also common (9).

As perceptual abnormalities most frequently affect the visual domain, several studies have examined the visual system in DLB patients with the aim of assessing potential structural and physiological changes that give rise to these phenomena. Although the causative factors are unknown, several hypotheses share the idea that the specificity and distribution of pathological alterations may be critical for the elicitation of visual hallucinations in DLB (10-12). Therefore, the manifestation of visual hallucinations in DLB may be related to the degeneration of some regions, but also the relative preservation of others.

Neuropathological studies of the retina in DLB have demonstrated abnormal proteinaceous inclusions (13) which may (14), or may not (15), be Lewy body-type pathology. Additionally, retinal nerve thinning (16) and electroretinogram abnormalities (17) have demonstrated potential functional changes in the retina in DLB. However, the lateral geniculate nucleus (LGN), the primary afferent visual relay structure between the retina and the primary visual cortex, is relatively spared in DLB compared to AD (18), suggesting that visual hallucinations in DLB may be facilitated by pathological changes in other parts of the visual system.

In DLB patients with visual hallucinations, focusing attention upon the object of hallucination has been demonstrated to promote its cessation (19), implying that visual attention may play a role in this phenomenon. Perceptual changes resulting from altered visual attentional processes have been postulated to contribute a vulnerability to hallucination in DLB (12, 20). The pulvinar nucleus of the thalamus plays a central role in visual attentional mechanisms (21, 22), and lesions to the pulvinar can cause deficits in filtering distracting stimuli (23) and feature binding of visual objects (24). The pulvinar has widespread cortical connections and is thought to play a general role in modulating cortico-cortical activity based on attentional demands (25). The pulvinar nucleus is traditionally parcellated into four anatomical sub-regions: anterior, medial, lateral and inferior (26). However, these histological sub-regions do not map perfectly onto sub-regions that have been segregated based on functionality or connectivity (27).

Recent evidence has suggested that the pulvinar undergoes degeneration in DLB (28) and that DLB cases have reduced metabolism in the pulvinar (29). The degree of degeneration in sub-regions of the pulvinar, as assessed by mean diffusivity on fMRI, has been demonstrated to predict clinical markers of visual hallucination frequency and severity (28). Despite these findings, no previous neuropathological study has examined the sub-regions of the pulvinar in DLB in the context of visual hallucinations. However, one study did report Lewy body pathology in the pulvinar as a whole, as part of a wider study of the visual system (30). The present study therefore aimed to investigate the sub-regions of the pulvinar in post-mortem tissue taken from DLB cases that had experienced visual hallucinations during life to assess potential degenerative morphometric and/or neuropathological changes using unbiased stereological methods.

METHODS AND MATERIALS

Tissue preparation

Human *post-mortem* tissue was obtained from the Newcastle Brain Tissue Resource (NBTR) and ethical approval was granted by Newcastle University ethics board and the Joint Ethics Committee of Newcastle and North Tyneside Health Authority (ref: 08/H0906/136). DLB and AD subjects had been part of several prospective clinical studies, and had received detailed clinical assessments and case note review after death. Neuropathological assessment was performed according to standardized neuropathological diagnostic procedures (31-35). Clinical and pathological data was collated to establish a clinico-pathological consensus diagnosis.

Three groups of cases were included in the present study: DLB cases that had experienced complex visual hallucinations during life, AD cases that had not experienced visual hallucinations during life and clinically confirmed aged control cases that showed none, or only low, age-associated neurodegenerative pathology at *post-mortem* examination.

At autopsy, the right hemisphere was fixed in 10% formalin and cut into 7 mm coronal slices, prior to further dissection into blocks for neuropathological assessment. The pulvinar nucleus was identified by its location at the posterior portion of the thalamus, ending in the lateral ventricle (36). Only cases containing the entire pulvinar were included for histological analyses, giving a group of eight control, eight DLB and eight AD cases. The pulvinar were exhaustively serially sectioned, with 30 μm and 10 μm sections obtained at each 1 mm interval. 30 μm sections were stained with cresyl fast violet for stereological analyses. 10 μm sections were stained with antibodies against a range of protein targets (Table 1) using Menarini Menapath Polymer detection kits (Menarini, Berkshire, UK), as per manufacturer's instructions.

Stereology

The border of the anterior and medial pulvinar could not be reliably differentiated through coronal examination so these regions were thus grouped together for stereological analyses and will subsequently be referred to as the ‘anteromedial pulvinar’. The inferior pulvinar was incomplete in almost every case due to its location at a point where the midbrain is dissected from the diencephalon, thus precluding stereological analysis of this sub-region. The lateral pulvinar was differentiated from other structures based on its striated appearance (37).

Stereological analysis was conducted using a Zeiss AxioVision Z.1 microscope equipped with a motorized stage (Zeiss, Oberkochen, Germany), coupled to a computer with Stereologer software (Bethesda, MA, USA).

Stereological estimates were established in the anteromedial and lateral pulvinar nuclei based on (37) as shown in figure 1. Volumes were determined in the anteromedial and lateral pulvinar nuclei using Cavalieri’s principle and mean cell densities within each nucleus estimated using the optical disector method (38).

Cavalieri’s principle was calculated by the following equation:

$$V \coloneqq T \cdot a \cdot \Sigma p$$

Cavalieri’s principle allows estimation of the total volume of each region of interest per case based on the intersection distance (T), the area per point (a) and the sum of the number of counted points (p). For estimation of volume, frames were placed in a uniform random manner, with disector frames spaced at 975 μm for anteromedial pulvinar, and 800 μm for lateral pulvinar, based on the relative size and distribution of the structures examined.

The rater (D.E.) traced an outline around the region of interest (i.e. anteromedial or lateral pulvinar) using a 2.5x objective. Disector frames were placed in a uniform, random arrangement to calculate the density of cells within a defined region, using the following equation:

$$Nv = \frac{\sum^{p-} Q^{-}}{P \cdot V}$$

Where Nv = numerical density, $p-$ = disector samples, Q^{-} = Q-weighted number of objects counted, P = total number of disectors, and V = disector volume.

Neuronal counts were conducted at 63x oil-immersion objective using the optical disector probe. Glial cell counts were calculated in both pulvinar sub-regions in disector frames of 3500 μm^2, with neuron counts calculated in disector frames of 1900 μm^2. Section thickness did not vary across disease groups in anteromedial or lateral pulvinar. The mean coefficients of error (CE) for neuronal and glial cell estimates was calculated using the Gundersen-Jensen method (39), as illustrated by the following equation:

$$CE^2 = \left(\frac{\Sigma\,(I^2)}{(\Sigma\,I)^2} + \frac{\Sigma\,(Volume^2)}{\Sigma\,(Volume^2)} - \frac{2\Sigma(1 \cdot Volume)}{(\Sigma\,I \cdot \Sigma\,Volume)}\right) \cdot \left(\frac{n}{n-1}\right)$$

Where I = neurons counted, $Volume$ = reference area x (sampling frame density)^2 x section depth, and n = number of fields.

Using the Gundersen-Jensen method (39), mean coefficient of error (CE) values for all stereologically-obtained data showed acceptable levels of accuracy (<0.10), with error values contributing less than 50% of the total observed coefficient of variance (CV). These values are considered to have acceptable levels of accuracy for stereological estimates (40).

Neuropathology

The anterior, medial and lateral nuclei of the pulvinar were analyzed using quantitative neuropathological techniques. Although the anterior and medial pulvinar border could not be discerned reliably for stereological analysis, where the entire structure along its antero-posterior extent is required, it was possible to identify the individual structures for analysis of neuropathology using one section per structure. For analysis of the anterior pulvinar, the section which contained the emergence of the anterior pole of the pulvinar was used. The medial and lateral pulvinar nuclei were defined as the region at which both structures were at their maximal area on coronal section, as in (41).

To quantify neuropathological lesions, images of the sub-nuclei of the pulvinar were taken on a Zeiss AxioVision Z.1 microscope using a DsFi1 camera (Nikon, Tokyo, Japan). Stereologer software was used to delineate a region of interest with a 2.5x objective, prior to placement of disector frames in a uniform, random arrangement. This method prevented the introduction of bias by giving every area of the region of interest an equal probability of being sampled for analysis. Disector frame sizes were determined based on the size of the measured particles and their distribution across the region of interest. In all cases, amyloid-β and tau were measured using 10x objective and α-synuclein, CD68 and glial fibrillary acidic protein (GFAP) were measured using 20x objective. Images were taken within the disector frames and analyzed using ImagePro Plus v.4.1 image analysis system (Media Cybernetics, Bethesda, MA, USA). Using previously published techniques (18, 42), the mean percentage area of immunopositivity was determined by standardizing red-green-blue (RGB) thresholds per antibody and applying to all sections per case. Each case thus had a mean value generated per antibody across all sections analyzed.

RESULTS

Demographics

No significant difference was found between groups in terms of age at death ($p=0.63$) or *post-mortem* delay ($p=0.43$; Table 2).

Final MMSE scores were available for 14/24 cases (five control, five DLB, four AD) and there was no significant difference between groups in the interval from last assessment to death ($p=0.36$). MMSE scores were significantly reduced in DLB ($p<0.01$) and AD ($p<0.01$) cases compared to controls, but there was no significant difference between AD and DLB (Table 2).

Stereology

In the anteromedial pulvinar, no significant main effect of diagnosis on neuronal ($F=3.02$, $p=0.07$) or glial number ($F=1.00$, $p=0.39$) was found, and the volume of the structure was not significantly different across groups ($F=1.27$, $p=0.30$; figure 2).

In the lateral pulvinar, a significant main effect of diagnosis on neuronal number was found ($F=14.219$, $p<0.01$). Post-hoc tests using Tukey's HSD showed a significant mean 30.7% decrease in neuronal number in DLB cases compared to controls ($p<0.01$) and a significant (16.7%) decrease in AD cases compared to controls ($p=0.02$). Despite DLB cases showing a greater degree of neuronal loss than AD cases, the differences were not statistically significant ($p=0.06$). No significant differences in glial cell number ($F=0.125$, $p=0.88$) or lateral pulvinar volume ($F=2.45$, $p=0.11$) were found across groups (figure 2).

Neuropathology

α-synuclein was significantly higher in DLB cases in all three regions analyzed compared to control and AD cases (fig. 3). In DLB cases, the medial pulvinar was, invariably, more severely affected by α-synuclein pathology than the lateral ($p=0.01$) or anterior nuclei ($p=0.05$), and no significant difference in expression was found between anterior and lateral nuclei (fig. 4). Again as expected, amyloid-β and tau pathology were significantly higher in AD cases when compared to control cases (fig. 3).

No significant differences were found between groups in the microglial marker CD68 in any pulvinar sub-region (data not shown). GFAP, a marker of astrocytes, was significantly increased in DLB and AD cases in all pulvinar sub-regions compared to control cases. However, there was no significant difference in GFAP expression between DLB and AD cases in any sub-region. There was a 47.3% increase ($p=0.01$) in DLB cases and a 57.2% increase ($p<0.01$) in AD cases compared to control in GFAP expression in the anterior pulvinar. There was a 46.9% increase ($p=0.04$) in DLB cases and a 56.6% increase ($p<0.01$) in AD cases compared to control in GFAP expression in the medial pulvinar. There was a 37.6% increase ($p=0.05$) in DLB cases and a 46.9% increase ($p=0.01$) in AD cases compared to control in GFAP expression in the lateral pulvinar.

DISCUSSION

The present study found a significant loss in the number of neurons in the lateral, but not anteromedial, pulvinar of DLB cases with visual hallucinations compared to controls. The lateral pulvinar of AD cases also showed a significant reduction in neuron number when compared to control cases and, whilst this reduction was less than that found between DLB and control cases, there was no significant difference between DLB and AD. Lewy body pathology and increased astrocyte immunoreactivity was also found in the pulvinar in DLB against control cases, but no significant difference in astrocyte expression was found in DLB compared to AD cases.

A previous study has shown the pulvinar is vulnerable to Lewy body pathology, in comparison to other visual regions of the thalamus, such as the LGN (30). This is in broad agreement with the findings outlined in this study, as well as our previous study in the LGN (18). Here, we extend these findings by demonstrating a specific pattern of neuronal loss in the lateral pulvinar, with the findings of no change in neuronal number in the anteromedial pulvinar mirroring our previous findings in the LGN. In contrast, AD cases had significant neuronal loss in the LGN (18) and less marked neuronal losses in the lateral pulvinar when compared to DLB. Taken together, these findings suggest that neuronal loss in the visual thalamus in DLB is specific to the lateral pulvinar, where neuronal loss is more severe than that observed in AD. This is an interesting finding as stereological studies conducted in regions outside of the mid-brain do not usually find neuronal reductions in DLB that exceed those in AD (for a review see (43)). Our findings may therefore indicate that the severe and specific neuronal loss in the lateral pulvinar in DLB may contribute to differences in the clinical phenotypes between DLB and AD.

The lateral pulvinar is known to receive predominant innervation from visual cortical areas (44) and to be functionally involved in regulating cortical activity in vision-related pathways (45). As the lateral pulvinar has been shown to have a strong regulatory influence on the functioning of the primary visual cortex (46), its degeneration in DLB may lead to altered functioning of the visual cortex, which, in turn, may contribute to hallucinogenesis. In primate visual area V4, deactivation of the lateral pulvinar leads to reduced frequency of cortical oscillations similar to those observed during inattention or sleep (21). Additional lesion studies of the lateral pulvinar in non-human primates have demonstrated behavioral changes indicative of perceptual neglect, such as reluctance to grasp target stimuli with the contralateral limb (47), indicating a role in visual attention. As impaired visual attentional function is thought to contribute to the manifestation of visual hallucinations in DLB (12, 20), the neuronal loss observed in the lateral pulvinar may relate to the occurrence of visual hallucinations through visual attentional impairment.

The medial pulvinar, which possessed the greatest α-synuclein pathology among the pulvinar nuclei examined in DLB cases (fig. 4), has been shown in non-human primates to have substantial reciprocal connectivity with regions known to be vulnerable to α-synuclein pathology, including the amygdala and cingulate gyrus (48). This is in contrast to the lateral pulvinar, which has greater connectivity with early visual cortical areas (22), which are often unaffected by Lewy body pathology in DLB (49). Similarly, the anterior pulvinar is substantially connected with the somatosensory cortex (50), which is only affected at late stages of Lewy body pathology (51). Considering the emerging view suggesting α-synuclein pathology may spread in a manner reminiscent of prion protein (52, 53), it is perhaps unsurprising that greater Lewy body pathology is observed in regions that are connected to sites severely affected and at early stages in the progression of DLB. However, it is possibly more surprising that the medial pulvinar did not exhibit neuronal loss in DLB, considering its higher burden of α-synuclein pathology. One possible explanation is that neuronal loss is the result of reduced input from regions that project to the lateral pulvinar, with reductions occurring over time as a result of diminished input.

Previous neuroimaging findings have shown that DLB cases have reduced grey matter density, as measured by mean diffusivity, in posterior thalamic regions that project to occipital and parietal regions (28). While the cytoarchitectonic parcellation of the pulvinar into anterior, medial, lateral and inferior sub-regions is not fully compatible with its segregation based on its physiology and connectivity (27), areas corresponding to the lateral pulvinar have been shown to project to occipital and parietal regions (44, 54). Hence our finding of neuronal loss in the lateral pulvinar corroborates neuroimaging studies by providing a neuropathological correlate for reduced grey matter density (28). Changes in mean diffusivity on neuroimaging have also been demonstrated to relate to clinical markers of visual hallucination frequency and severity, suggesting a relationship between degeneration of pulvinar sub-nuclei that project to occipital regions and the occurrence of visual hallucinations (28).

The specific pattern of neuronal loss seen in the lateral pulvinar in DLB patients has also been demonstrated in stereological studies of schizophrenic patients (55). Although visual hallucinations are relatively uncommon in schizophrenia (56), schizophrenic and DLB patients have visual attentional deficits (9, 57) and impairments in smooth pursuit eye movements (9, 58), which can occur as a result of attentional dysfunction (59). Considering the putative role of the lateral pulvinar in modulating visual cortical activity based on attentional demands (21, 45, 46), these findings may highlight a common degenerative change that promotes visual attentional dysfunction in both disorders. In DLB, visual attentional deficits may act in concert with dysfunction or degeneration of other brain regions to elicit hallucinations.

In summary, we have shown specific patterns of degeneration in the pulvinar of DLB cases and that these degenerative changes are more severe in DLB compared to AD. The putative role of the lateral pulvinar in modifying the response properties of visual cortical neurons based on attentional demands might suggest that lateral pulvinar degeneration contributes to deficient visual attentional mechanisms and corresponding cortical activity changes, which have both previously been related to visual hallucinations in DLB (12, 60). The results of our current study support neuroimaging findings associating the degeneration of particular pulvinar sub-regions with visual hallucinations in DLB (28). However, it should be noted that the pulvinar is one component in a highly complex and incompletely understood system and is therefore likely to act in concert with other regions to contribute to the occurrence of visual hallucinations. Hence, continued study of the vulnerability of the visual system is warranted to further our understanding of the pathological changes that promote visual hallucinations in DLB.

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TABLES AND LEGENDS

Table 1: Antibody dilutions.

Antibody	Manufacturer	Dilution	Antigen retrieval
GAD65/67	Sigma	1:6000	Citrate pH 6
4G8 amyloid-β	Covance	1:15,000	Formic acid
AT8 phosphorylated-τ	Autogen	1:10,000	Citrate pH 6
5G4 α-synuclein	Analytik Jena	1:4500	Citrate pH 6 + formic acid

Table 2: Demographic characteristics of cohort. 'Age' refers to age at death, 'PM delay' refers to the delay between death and post-mortem examination, 'Braak NFT' stage is neurofibrillary pathology stage outlined in (61), 'Thal phase' is amyloid-β pathology stage as outlined in (33), 'McKeith Lewy body stage' is Lewy body pathology stage outlined in (32), 'MMSE' is mini-mental state examination score, 'NA' represents data not being available.

Case ID	Age	PM delay	Diagnosis	Braak NFT stage	Thal phase	McKeith Lewy body stage	Final MMSE
1	99	5	Control	2	0	None	27
2	77	83	Control	2	3	None	NA
3	85	57	Control	3	4	None	29
4	80	16	Control	2	0	Brainstem	29
5	65	47	Control	1	0	None	NA
6	73	25	Control	0	0	None	30
7	76	86	Control	2	1	Amygdala	30
8	78	23	Control	2	1	None	NA
9	73	99	DLB	3	4	Neocortical	13
10	81	81	DLB	3	4	Neocortical	NA
11	89	88	DLB	3	2	Neocortical	14
12	77	46	DLB	3	0	Neocortical	12
13	81	44	DLB	4	3	Neocortical	NA
14	78	96	DLB	3	3	Neocortical	9
15	91	10	DLB	3	4	Neocortical	NA
16	73	47	DLB	3	1	Neocortical	22
17	81	73	AD	5	5	None	NA
18	89	61	AD	6	5	None	18
19	68	24	AD	6	5	None	NA
20	85	32	AD	5	5	None	15
21	76	6	AD	6	5	None	6
22	86	123	AD	6	5	Brainstem	NA
23	95	23	AD	6	5	Amygdala	NA
24	85	39	AD	6	4	None	19

FIGURES AND LEGENDS

Figure 1: The anatomy of the pulvinar. The anteromedial (dashes) and lateral (dots) pulvinar nuclei are shown. Scale bar = 3 mm.

*Figure 2: Stereological estimates of number and volume in the pulvinar nuclei. *p<0.05, **p<0.01.*

*Figure 3: Quantitative neuropathology in the pulvinar sub-nuclei. *p<0.05 compared to control; **p<0.05 compared to control and DLB; ***p<0.05 compared to control and AD.*

Figure 4: α-synuclein pathology in the pulvinar sub-nuclei. Representative 5G4 staining in the (A) anterior, (B) lateral and (C) medial pulvinar of a DLB case. Scale bar = 50 µm.